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Antimicrobial peptide AMPNT-6 from *Bacillus subtilis* inhibits biofilm formation by *Shewanella putrefaciens* and disrupts its preformed biofilms on both abiotic and shrimp shell surfaces

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Abstract

Shewanella putrefaciens biofilm formation is of great concern for the shrimp industry because it adheres easily to food and food-contact surfaces and is a source of persistent and unseen contamination that causes shrimp spoilage and economic losses to the shrimp industry. Different concentrations of an antimicrobial lipopeptide, the fermentation product of *Bacillus subtilis*, AMPNT-6, were tested for the ability to reduce adhesion and disrupt *S. putrefaciens* preformed biofilms on two different contact surfaces (shrimp shell, stainless steel sheet). AMPNT-6 displayed a marked dose- and time-dependent anti-adhesive effect > biofilm removal. 3MIC AMPNT-6 was able both to remove biofilm and prevent bacteria from forming biofilm in a 96-well polystyrene microplate used as the model surface. 2MIC AMPNT-6 prevented bacteria from adhering to the microplate surface to form biofilm for 3 h and removed already existing biofilm within 24 h. Secretion of extracellular polymeric substances incubated in LB broth for 24 h by *S. putrefaciens* was minimal at 3× MIC AMPNT-6. Scanning electron microscopy showed that damage to *S. putrefaciens* bacteria by AMPNT-6 possibly contributed to the non-adherence to the surfaces. Disruption of the mature biofilm structure by AMPNT-6 contributed to biofilm removal. It is concluded that AMPNT-6 can be used effectively to prevent attachment and also detach *S. putrefaciens* biofilms from shrimp shells, stainless steel sheets and polystyrene surfaces.

Keywords: *Bacillus subtilis*; antimicrobial lipopeptide; *Shewanella putrefaciens*; anti-adhesion; biofilm removal

1. Introduction

Shewanella putrefaciens, a marine, Gram-negative bacterium commonly found in both water and soil, is a specific spoilage organism (SSO) of even chilled *Litopenaeus vannamei* and other aquatic organisms under aerobic conditions (Leblanc, Leboeuf, Leroi, Hartke, & Auffray, 2003; Vogel, Venkateswaran, Satomi, & Gram, 2005). *S. putrefaciens* contamination of shrimp products affects the economy of the shrimp industry. It can also cause corrosion of processing equipment and packaging materials and can cause metals [e.g. Fe(III) and Mn(IV)] and organic material to disintegrate because of the ability of *S. putrefaciens* to produce volatile sulfides, amines, and the "fishy-smelling" compound trimethylamine (Bagge, Hjelm, Johansen, Huber, & Gram, 2001; Dawood & Brozel, 1998). In addition, *S. putrefaciens* is able to adhere to various solid surfaces and form biofilms (Bagge, Hjelm, Johansen, Huber, & Gram, 2001). Once a biofilm is formed on a contact surface it is difficult to remove and therefore is a potential source of food contamination that can lead to a short shelf-life and health risk to susceptible consumers (Lebet, Leroy, & Talon, 2007). Therefore, there is a need to find an efficient way to control biofilm formation by *S. putrefaciens.*

In the past decade, several strategies have been used to control biofilm formation. Chemical and physical methods have many disadvantages, such as high cost, operational difficulties, health hazards and resistance development (del Pozo et al., 2009; Schlag, Birkenstock, Altenberend, & Gotz, 2007; Ziech et al., 2016). In contrast, biological methods, which are now more widely studied, are considered generally safe, with no harmful residues in food or antibacterial resistance (Gomes & Nitschke, 2012;

Raaijmakers, de bruijn, Nybroe, & Ongena, 2001). Biological methods have limitations, such as cost, because natural biological antibacterial preparations are expensive and relatively unstable. Antimicrobial lipopeptides are a new type of alkaline polypeptides that exhibit excellent broad-spectrum antimicrobial properties and hence are good candidates for application in the food industry to control biofilm formation (Gomes & Nitschke, 2012; Raaijmakers, de bruijn, Nybroe, & Ongena, 2001). Currently, nisin is widely used as a biofilm control agent for Gram-positive (G^+) bacteria in acidic environments (de Arauz, Jozala, Mazzola, & Penna, 2009). However, most SSOs are Gram negative (G^-) and aquatic products are usually in a neutral or alkaline environment; therefore, the control of biofilm formation by SSOs in aquatic products has become problematic (Norhana, Poole, Deeth, & Dykes, 2012). *Bacillus subtilis* is a non-pathogenic bacterial strain, and its fermentation products are the AMPNT-6 lipopeptides surfactin, iturin and fengycin. AMPNT-6 has an amphipathic structure with a hydrophobic fatty acid section and a hydrophilic peptide section. The molecular structures of these lipopeptides are cyclic, because C-terminal peptide residues connect either with β-hydroxy fatty acid through the hydroxyl group of the peptide residue or directly with a β-amino acid (Jasim, Sreelakshmi, Mathew, & Radhakrishnan, 2016). AMPNT-6 has antimicrobial activity within a wide pH range of 2–12. It is thermally stable (even at 100 °C for 20 min), exhibits excellent two-phase solubility in both oil and water, and is effective not only against both G^+ and G^- bacteria but also against fungi (Banat et al., 2010; Sun, Lu, Bie, & Yang, 2006; Zhang et al., 2017). In this study, the antimicrobial lipopeptide AMPNT-6 was extracted from a *Bacillus subtilis* NT-6

fermentation broth and its effect evaluated for (1) reducing the biofilm-forming ability, and (2) removing already preformed biofilm, of *S. putrefaciens* SL-3, to provide a theoretical basis for practical industry application including controlling biofilm formation on food processing equipment.

2. Materials and methods

2.1. Microorganisms

Bacillus subtilis NT-6 (GeneBank: JN180628) and *Shewanella putrefaciens* SL-3 were obtained from the Food-borne Pathogenic Microorganisms and Toxins of Aquatic Products Green Control Laboratory, Food Science and Technology College, Guangdong Ocean University, Zhanjiang, China. *S. putrefaciens* SL-3 was isolated from spoiled *Litopenaeus vannamei* and incubated in Luria-Bertani (LB) broth (Land Bridge, Beijing) at 30 °C for 24 h as a bacterial suspension**,** and the concentration of *S. putrefaciens* suspension was measured to be 10^9 CFU mL^{-1}.Then 1 mL of *S. putrefaciens* suspension was pipetted into 9 mL of sterile distilled water to adjust it to 10^8CFU mL^{-1}.

2.2. Preparation of AMPNT-6

Bacillus subtilis NT-6 maintained on a slant was inoculated into LB liquid medium and cultivated at 37 °C with shaking at 150 r min^{-1} for 24 h for seed preparation. Then 5% (v/v) of the seed solution was added to 50 mL of Landy medium (glucose 20.0 g, sodium hydrogen L-glutamate 5.0 g, $MgSO_4·7H_2O$ 0.5 g, KCl 0.5 g, $KH_2PO_4·3H_2O$ 1.0 g, $FeSO_4·7H_2O$ 0.15 mg, $MnSO_4·H2O$ 5.0 mg, $CuSO_4·5H_2O$ 0.16 mg, distilled water 1000 mL, pH 7.2) in a 250-mL Erlenmeyer flask and cultivated at 30 °C, 160 r min^{-1} for

36 h, to produce AMPNT-6. After incubation, the fermentation broth was centrifuged at 10,000 r min^{-1} for 10 min to remove cells. The supernatant was subjected to acid precipitation by adding 6 M HCl up to pH 2, and then stored at 4 °C. After 12 h storage, the stored product was centrifuged at 5000 r min^{-1} for 15 min and the precipitate dissolved in anhydrous methanol. The pH was adjusted to 7.0 with addition of 1 M NaOH and the solution centrifuged at 10,000 r min^{-1} for 20 min at 4 °C. The AMPNT formed was concentrated by using a rotary evaporator (Eyela, N1000, China) under vacuum and quantitatively analysed using HPLC-MS (Deng et al., 2016). The minimum inhibitory concentration (MIC) of AMPNT-6 on *S. putrefaciens* SL-3 was determined as 0.3125 mg mL^{-1} by the double-dilution method (Fazeli et al., 2007).

2.3. Effects of AMPNT on adhesion of *Shewanella putrefaciens*

Preparation of contact media

Muscle tissue was removed from the shells of fresh *Litopenaeus vannamei* and the shrimp shells cut into chips of approximately 1 × 3 cm. The shell chips were rinsed with distilled water, immersed in 75% alcohol for 20 s, washed with sterile water and dried in a sterile environment.

Strips of stainless steel sheet (304L, 1 × 3 × 0.08 cm, bright annealed) were soaked overnight with 1M NaOH, rinsed with sterile distilled water, and then placed in acetone for 1 h (Scott & Edmund, 1997). The strips were then dried in a sterile environment.

Biofilm formation

The shell chips and stainless steel strips were then placed separately in test tubes containing 5 mL LB and *S. putrefaciens* suspension (final concentration of 10^6 CFU

mL^{-1}). Next, 100 μL of AMPNT-6 were added to the tubes to reach final concentrations of 0, 0.25, 0.5, 1.0, 2.0, 3.0 MIC, in triplicate for each contact medium, and cultured at 30 °C without agitation for 60 h.

Quantification of biofilm

After treatment, the shell chips and stainless steel strips were gently rinsed three times with sterile PBS, transferred to new sterile test tubes and 5 mL of physiological saline added. Ultrasonic oscillation (PS-60A, Jiekang Science and Technology (Hong Kong) Co., China) at a frequency of 40 KHz for 10 min, as described by Yang, Han, Chen, Pang, and Duan (2007), was performed to disperse biofilm bacteria in order to make a uniform cell suspension. The physiological saline containing biofilm bacteria was collected and 10-fold dilutions used for a plate count on nutrient agar media following incubation at 30 °C for 24 h, to quantify the bacteria in the biofilm. Results were expressed as Log CFU cm^{-2}.

2.4. Removal of biofilm by AMPNT-6

Shrimp shell chips and stainless steel strips were prepared as described above, placed in test tubes containing 5 ml LB and *S. putrefaciens* suspension (final concentration of 10^6 CFU mL^{-1}), and incubated for 60 h at 30 °C. AMPNT-6 was then added to the tubes to final concentrations of 0, 0.25, 0.5, 1.0, 2.0, 3.0 MIC, in triplicate, and cultured at 30 °C without agitation for 24 h. Ultrasonic oscillation as described above for shell chips and stainless steel strips was employed to quantify the *S. putrefaciens* biofilm at each treatment.

2.5. Scanning electron microscopy

The biofilm samples on the stainless steel strips obtained in section 2.3 and 2.4 (adhesion and removal experiments above) were imaged using an SEM (Sirion 200, Philips Co., The Netherlands). The samples were subjected to the processing method described by Gomes and Nitschke (2012). Briefly, the samples were washed three times with distilled PBS to remove the planktonic cells, fixed overnight with PBS solution containing 2.5% glutaraldehyde, rinsed three times with PBS solution, dehydrated using an ethanol series (50%, 70%, 80%, 90%) each for 10 min, dehydrated with 100% ethanol twice for 15 min each, exposed to isoamyl acetate twice for 15 min each, and dried in a critical-point dryer (Bal-tecCPD 030, Bal-tecTM, Los Angeles, CA).The stainless steel sheets with biofilm were spluttered with gold and visualised by SEM operating at 20 kV.

2.6. Anti-biofilm-forming kinetics on exposure to AMPNT-6

To 96-well polystyrene microplates (Corning Inc., New York, NY, USA) 150 µL of LB broth and 50 µL of *S. putrefaciens* suspension with 10^6 CFU mL^{-1} were added. Next, 20 µL of different AMPNT-6 concentrations (0, 0.25, 0.75, 1.0, 2.0 MIC) were added, in hexaplicate, and incubated at 30 °C for different time intervals (3, 6 12, 24, 30, 36, and 48 h). Biofilm quantification was performed according to the protocol of Stepanovic, Vukovic, Dakic, Savic, and Svabic-Vlahovic (2000). Briefly, following each incubation period, the suspension was removed, wells washed with PBS three times, fixed with 200 µL of methanol for 15 min, and stained for 15 min with crystal violet 1.0% (w/v). Next,

the wells were washed and 200 µL of 33% (v/v) acetic acid was added. The kinetics of adhered cells was determined by measuring the optical density at 630 nm using a Varioskan Flash (Model 3001, Thermoplate, USA).

2.7. Biofilm removal kinetics by AMPNT-6

To 96-well polystyrene microplates, 150 µL LB broth and 50 µL *S. putrefaciens* suspension of 10^6 CFU mL^{-1} were added and incubated at 30 °C for 24 h to form a biofilm. Cell suspension was then removed and the wells washed three times with 200 µL of PBS. Next, 200 µL of AMPNT-6 were added to the wells to achieve a final concentration of 0, 0.25, 0.5, 0.75, 1.0, 2.0 MIC, in hexaplicate, and incubated at 30 °C. The effectiveness of AMPNT-6 in removal of biofilms was examined at 3, 6 12, 24, 30, 36 and 48 h of contact with the biofilms. The kinetics of biofilm removal was quantified by measuring the optical density at 630 nm using a Varioskan Flash (3001, Thermoplate, USA) as above.

2.8. Quantification of extracellular polymeric substances (EPS)

To test tubes (n = 12) were added 5 mL LB broth containing 1% (v/v) *S. putrefaciens* bacterial suspension (final concentration 10^7 CFU mL^{-1}) and 100 µL of AMPNT-6 to achieve final concentrations of 0, 0.25, 0.5, 1.0, 2.0, and 3.0 MIC in duplicate and cultured at 30 °C without agitation for 24 h. Triplicate samples from each AMPNT-6 concentration were taken at each of four time intervals (3, 6, 12, 24 h). The EPS were quantified as described by Croxatto, Lauritz, Chen, and Milton (2007). Briefly, 4 mL of bacterial suspension from the above reaction mixes were centrifuged at

2000 r min^{-1} for 15 min and approximately 300 µL of supernatant collected. To the 300 µL of supernatant, 1200 µL of alcian blue stain was added, mixed and incubated at room temperature for 1 h. The mixture was next centrifuged at 8000 r min^{-1} for 10 min. All of the suspension was carefully removed, 1200 µL ethanol was added, gently mixed and centrifuged at 8000 r min^{-1} for 10 min. The precipitate obtained was suspended with 2 mL SDS and the OD_{620} measured using a 722S spectrophotometer (Shanghai Precision Scientific Instruments Co., China).

2.9. Statistical analyses

All experiments were carried out in triplicate unless otherwise stated. The results are expressed as mean ± standard deviation. The data were analysed by ANOVA to compare the treated groups with the controls (0 MIC), and the means of the different treatment doses were compared using Tukey's test, with $P < 0.05$.

3. Results

3.1. Effect of AMPNT-6 on adhesion and biofilm persistence

As described in the Methods, a counting method was performed to evaluate the anti-biofilm-forming and biofilm removing effect of AMPNT-6 on shrimp shell. A significant decrease in the number of attached/biofilm bacteria was observed when the bacteria and the biofilm were incubated even at a low AMPNT-6 concentration of 0.25 MIC compared with the control group ($P < 0.05$) (Fig. 1). The efficiency of anti-adhesion and biofilm detachment was greater at higher AMPNT-6 concentrations with the effect on anti-adhesion better than on removal. A low bacterial count of 0.4 Log

CFU cm^{-2} and hence incomplete detachment was evident at 3.0 MIC AMPNT-6 treatment. There was no colony growth on the plates at 3.0 MIC AMPNT-6.

[Fig. 1 about here]

AMPNT-6 also controlled the formation and the disruption of biofilm on stainless steel sheet effectively, with a highly significant ($P < 0.01$) reduction even at 0.25 MIC (Fig. 2). It displayed a similar effect to that observed on shrimp shells. A low number of attached bacteria (~ 0.5 Log CFU cm^{-2}) was observed when AMPNT-6 was applied at 2.0 MIC. No biofilm bacteria were evident on the 3.0 MIC AMPNT-6 plates.

[Fig. 2 about here]

3.2 Changes in biofilm assembly

Figure 3 shows the effect of AMPNT-6 treatment on attachment of *S. putrefaciens* SL-3 to stainless steel. It shows that EPS form a covering layer between the surface and bacteria and also intertwine to form a relatively dense net with pores and cavities representing a mature biofilm (Fig. 3A:b). The effect of AMPNT-6 was dose dependent with less bacteria adhered to the surface and no biofilm seen at higher AMPNT-6 concentrations. Some cellular breakages were present in the test groups 0.5, 1.0, and 2.0 MIC (Fig. 3A:c–e). No bacterial aggregation was observed on the surface of stainless steel strips when cultured in LB medium containing 3.0 MIC AMPNT-6 (Fig. 3A:f).

[Fig. 3 about here]

A mature biofilm (after 24-h contact) is a holistic structure with intertwined EPS (Fig. 3B:b). The integrity of the biofilm structure was destroyed on exposure to 0.5 MIC and 1.0 MIC AMPNT-6 (Fig. 3B:c–d). Planar forms and a smaller biofilm with larger

pores and damaged structure were observed on the surface at 24-h contact with 2.0 MIC AMPNT-6 (Fig. 3B:e). At 3.0 MIC AMPNT-6, no biofilm was evident (Fig. 3B:f).

3.3 Anti-biofilm-forming and biofilm removal kinetics

The anti-adhesion effect of different concentrations of AMPNT-6 showed a similar dose-response effect at 48 h of incubation (Fig. 4). A significant dose-dependent reduction in biofilm biomass was observed due to the addition of AMPNT-6 between 6 and 24 h of incubation and the OD_{630} value of biofilm remained stable from then onwards.

[Fig. 4 about here]

AMPNT-6 did not cause an effective reduction on preformed biofilms till 12 h of incubation and there was no significant difference ($P > 0.05$) between the experimental groups (excluding the control group) during this period (Fig. 5). However, disruption to biofilm occurred following 12 h of incubation in the presence of all tested concentrations of AMPNT-6. The increase in concentration of AMPNT-6 increased the removal of biofilms significantly ($P < 0.05$) compared with the control group (0 MIC), and almost all the biofilms were eliminated at 2.0 MIC after 30-h contact.

[Fig. 5 about here]

3.4 Effect of AMPNT-6 on secretion of EPS by *Shewanella putrefaciens*

The quantity of EPS increased on exposure to 0.5 and 1.0 MIC AMPNT-6 addition during the initial 6 h, then decreased significantly at 12 h and remained at a low level (at about 0.11 OD_{620}) in the following 12 h (Fig. 6). Only a low EPS production of about

0.1 OD_{620} value was observed on contact at 2.0 and 3.0 MIC AMPNT-6. AMPNT-6 at 3.0 MIC significantly inhibited EPS production and remained < 0.1 OD_{620} at 12- and 24-h contact.

[Fig. 6 about here]

4. Discussion

Bacillus subtilis is known to produce a wide variety of structurally diverse secondary metabolites with immense potential for biotechnological, biocontrol and therapeutic applications via the non-ribosomal peptide synthetases pathway (Loiseau et al., 2015; Pu et al., 2013). Previously, cyclic lipopeptides of the surfactin, iturin and fengycin families were identified as the main components in the *B. subtilis* fermentation broth (Zhang et al., 2017). It is well established that different bacterial strains produce different antimicrobial lipopeptides with different efficacies. The MIC of AMPNT-6 antimicrobial activity to *S. putrefaciens* SL-3 was 0.3125 mg mL^{-1}. It is known that *S. putrefaciens* is susceptible to antimicrobial lipopeptides from *Bacillus amyloliquefaciens* ES-2 with a MIC of 0.6 mg mL^{-1} (Liu, Sun, Wang, Lei, & Xu, 2012). In that respect, AMPNT-6 is about two-fold more efficient. Lipopeptides act by detaching bacteria and by disruption of biofilms. Surfactin adversely affects the attachment of *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella* Enteritidis to polystyrene microplate surfaces on incubation for 25 h (Gomes & Nitschke, 2012). In this study, AMPNT-6 inhibited the adherence of *S. putrefaciens* SL-3 to polystyrene surfaces at incubation as short as 12 h. Unlike in the present study reported here, an increase in surfactin concentration does not significantly increase the

removal of biofilms, and a shorter contact period (12 h) is more effective in biofilm disruption than longer periods (Gomes & Nitschke, 2012). Some results of this study reported here are in contradiction with the literature. Such discrepancies could be explained by the different test strains, experimental conditions, and different lipopeptides used.

Antimicrobial lipopeptide surfactin is able to remove *Legionella pneumophila* biofilm but is not efficient at preventing adhesion (Loiseau et al., 2015). In contrast, Mireles, Toguchi, and Harshey (2001) showed that surfactin is better at preventing adhesion than in biofilm removal. In the present study, AMPNT-6, which contains not only surfactin, but also two other antimicrobial lipopeptides, iturin and fengycin, was efficient in both anti-adhesion and biofilm removal.

Surfaces of food processing equipment are usually made from stainless steel. From a food safety aspect, an overlooked contact surface is shrimp shell during shrimp processing (Bagge, Hjelm, Johansen, Huber, & Gram, 2001; Han, Mizan, Jahid, & Ha, 2016; Raaijmakers, de bruijn, Nybroe, & Ongena, 2001). *S. putrefaciens* has been shown to form biofilm on the surfaces of both stainless steel and shrimp shells and yet there are only a few reports that have focused on controlling biofilm on these surfaces. Because initial bacterial cell attachment is to solid surfaces, the properties of such surfaces will influence biofilm formation (Shi & Zhu, 2009). For example, Jones, Adams, Zhdan, and Chamberlain (1999) noted that surface roughness significantly increased biofilm production. It has been shown that stainless steel chips were more conducive for biofilm formation of *Vibrio cholarae* O1 than shrimp chips (Han, Mizan,

Jahid, & Ha, 2016). The results of this study are in agreement in that ability of *S. putrefaciens* SL-3 to form biofilms on stainless steel chips observed was more effective than on shrimp shells in this study. Therefore, it was more difficult for AMPNT-6 to prevent adhesion and remove biofilm from stainless steel chips than from shrimp shells. Lipopeptides may be able to change the chemical character of contact surfaces, which is one of the most important factors that affect biofilm formation, and in our study AMPNT-6 was effective in controlling *S. putrefaciens* SL-3 biofilm formation and is in agreement with the study of Han, Mizan, Jahid, & Ha (2016).

Bacteria can secrete EPS even in the absence of abiotic surfaces (Flemming, Neu, & Woznika, 2007). EPS is the main component of the biofilm responsible for adhesion (Chen & Stewart, 2000; Croxatto, Lauritz, Chen, & Milton, 2007) and can strengthen interactions between bacterial cells and thus influence the process of bacterial colony formation on contact surfaces (Branda, Vik, Friedman, & Kolter, 2005). In our study, the amount of EPS was increased within the first 6 h at low AMPNT concentration (0.5 and 1.0 MIC) but significantly decreased thereafter (Fig. 6). This phenomenon is most likely to have occurred because bacteria were not killed at such low AMPNT-6 concentrations even though the amount of EPS increased during the bacterial exponential growth phase (Voberkova, Hermanova, Hrubanova, & Krayzanek, 2016). Therefore, an inference could be drawn that AMPNT-6 was able to suppress biofilm formation by inhibiting the production of EPS. Furthermore, it was apparent from our study that the bacterial cell was the main attack target. This resulted in a reduction in the number of viable cells on addition of AMPNT-6, as shown by the damaged bacterial

cells and less cells adhered to stainless steel sheets as observed in SEM (Fig. 3A). Thus when the bacteria were cultured with AMPNT-6, anti-adhesion played an important role in preventing biofilm formation. In addition, AMPNT-6 also damaged the preformed biofilm structure (Fig. 3B) thus allowing AMPNT-6 to permeate into an incomplete biofilm and inhibit bacterial growth, thereby making the biofilm weak and ultimately causing death and removal of biofilm bacteria from the surfaces (Ceotto-Vigoder et al., 2016). Results from these studies indicate that the effect of AMPNT-6 on the cell and EPS occur simultaneously but the target may be different at different stages.

AMPNT-6 displayed a marked anti-adhesive effect > biofilm removal. This is probably due to two factors: (1) The growth and multiplication of the test strain is inhibited, which leads to a low concentration of this strain in the LB culture, and thereby prevent efficient adherence of the strain to the surface; (2) Once a mature biofilm with enveloping EPS is formed, it is more difficult for AMPNT-6 to permeate into the dense and reticular structure of the biofilm (Kuiper et al., 2004).

5. Conclusion

The present study clearly showed that AMPNT-6 from *Bacillus subtilis* NT-6 possesses strong activity against *S. putrefaciens* SL-3, which is the SSO of shrimp. EPS could be inhibited by AMPNT-6. AMPNT-6 was able to efficiently control biofilms on different contact surfaces by reducing adhesion of and/or removing biofilm. AMPNT-6 effects on biofilm adhesion and removal were dose- and time-dependent.

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Compliance with ethical standards This study was conducted according to the Guangdong Ocean University highest ethical laboratory standards.

Conflict of interest The authors declare they have no conflicts of interest.

Human and animal rights No procedures in this study, performed by any of the authors, involved human or animal participants.

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Figure Captions

Fig. 1. Evaluation of AMPNT-6 as an agent to reduce adhesion and remove *Shewanella putrefaciens* SL-3 biofilm on shrimp shell chips.

Fig. 2. Evaluation of AMPNT-6 as an agent to reduce adhesion and remove *Shewanella putrefaciens* SL-3 biofilm on stainless steel strips.

Fig. 3. Scanning electron micrograph images of *Shewanella putrefaciens* SL-3 biofilm formed on stainless steel strips. (A) AMPNT-6 was added and incubated to 60 h: (a) Without AMPNT-6 and bacterial addition, and (b–f) with 0, 0.25, 0.5, 1.0, 2.0, 3.0 MIC AMPNT-6 addition respectively (magnification 4000×). The arrows (c–f) indicate some cells with breakage. (B) AMPNT-6 was added to contact surface with a 60 h biofilm for 24 h: (a) Without AMPNT-6 and bacterial addition and (b–f) with 0, 0.25, 0.5, 1.0, 2.0, 3.0 MIC AMPNT-6 addition respectively (magnification 2000×).

Fig. 4. Anti-biofilm-forming kinetics of *Shewanella putrefaciens* SL-3 on polystyrene surfaces of microtiter plates conditioned with AMPNT-6.

Fig. 5. Biofilm removal kinetics of *Shewanella putrefaciens* SL-3 on polystyrene surfaces of microtiter plates conditioned with AMPNT-6.

Fig. 6. Effect of AMPNT-6 on the ability of *Shewanella putrefaciens* SL-3 to produce extracellular polymeric substances (EPS).

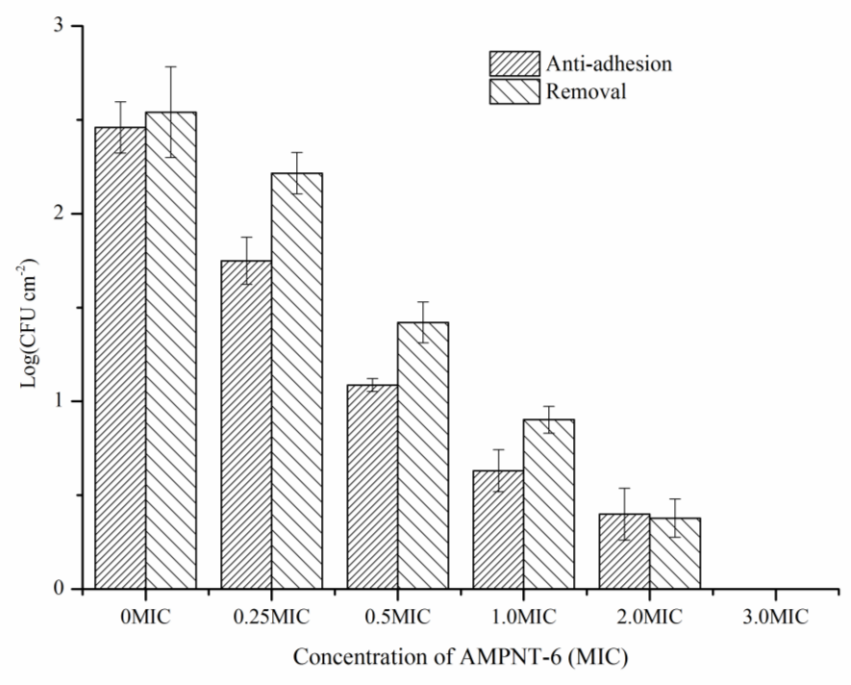


Fig. 1

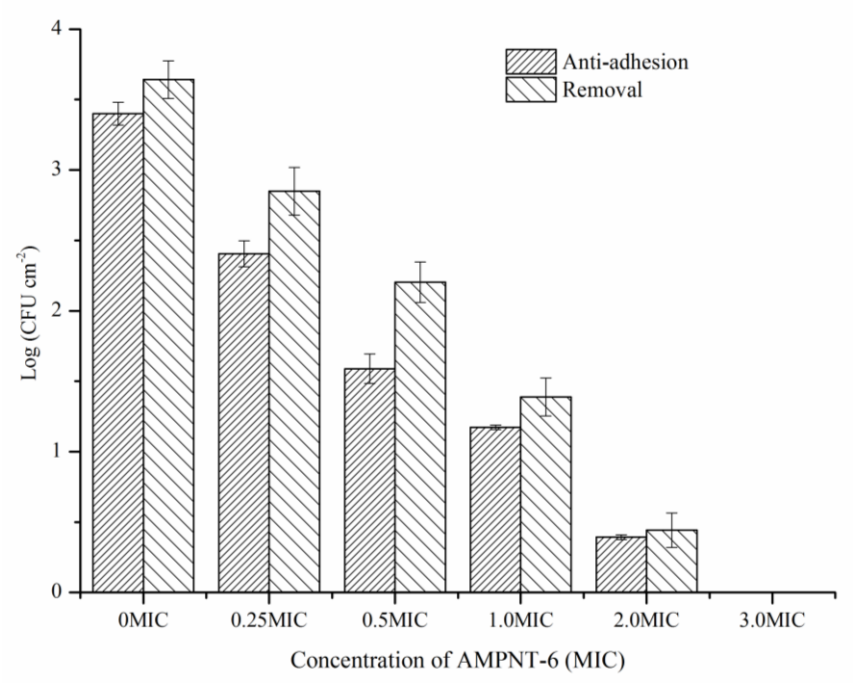


Fig. 2

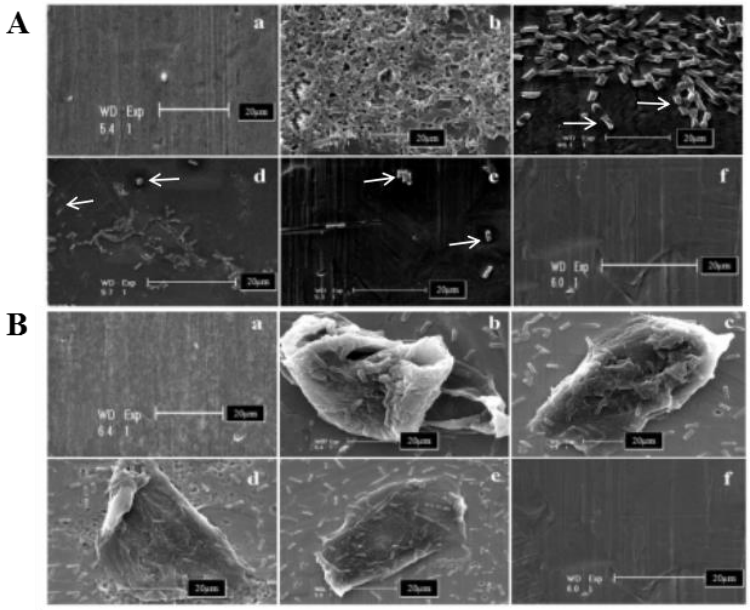


Fig. 3

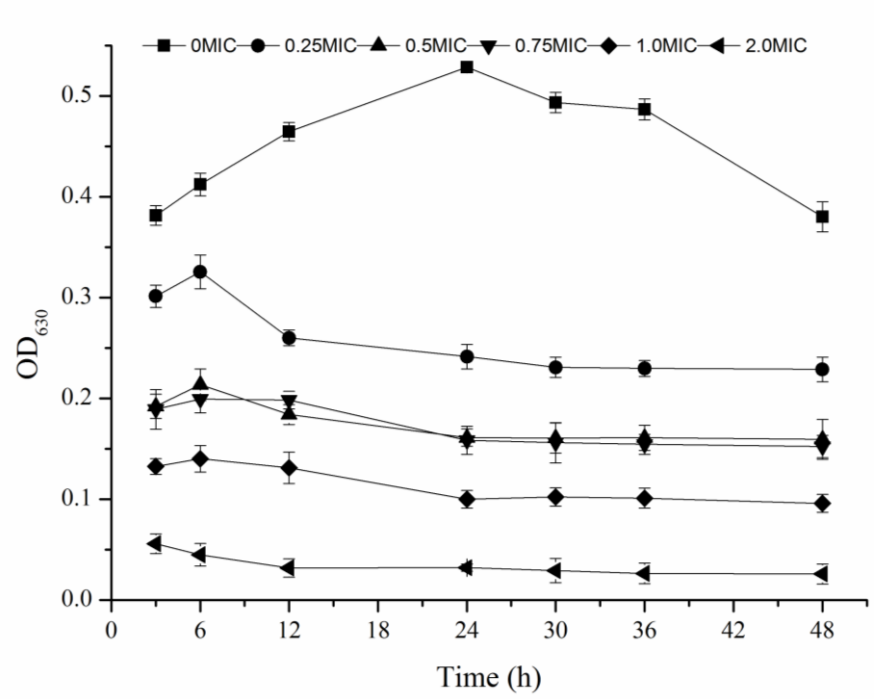


Fig. 4

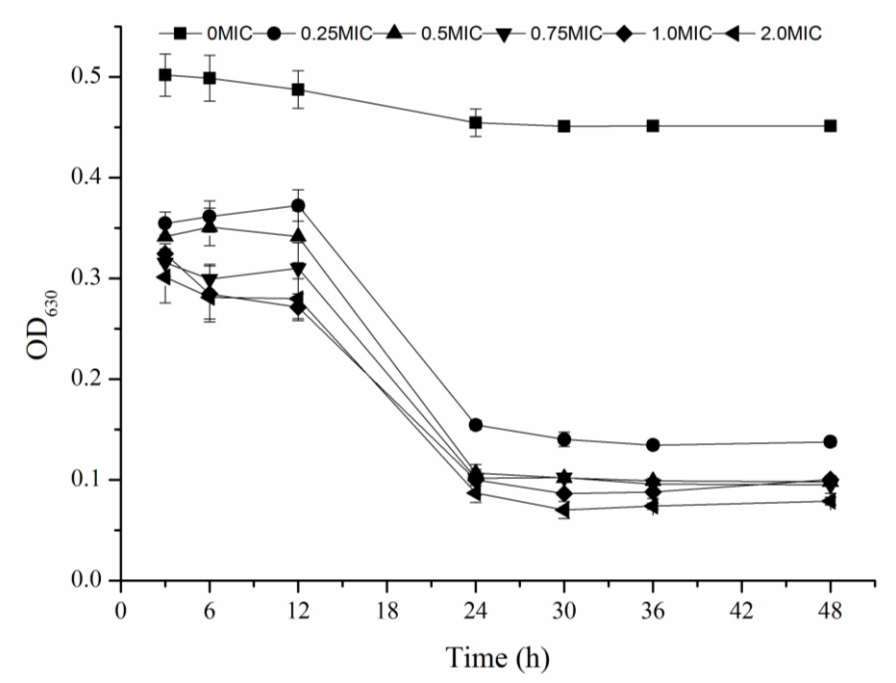


Fig. 5

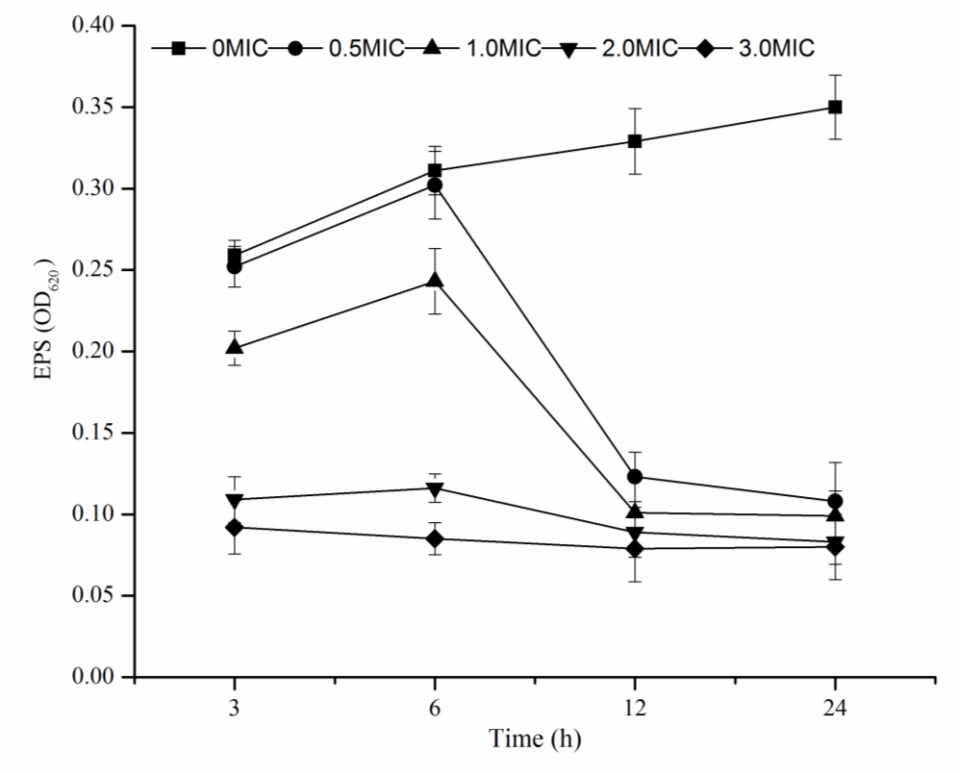
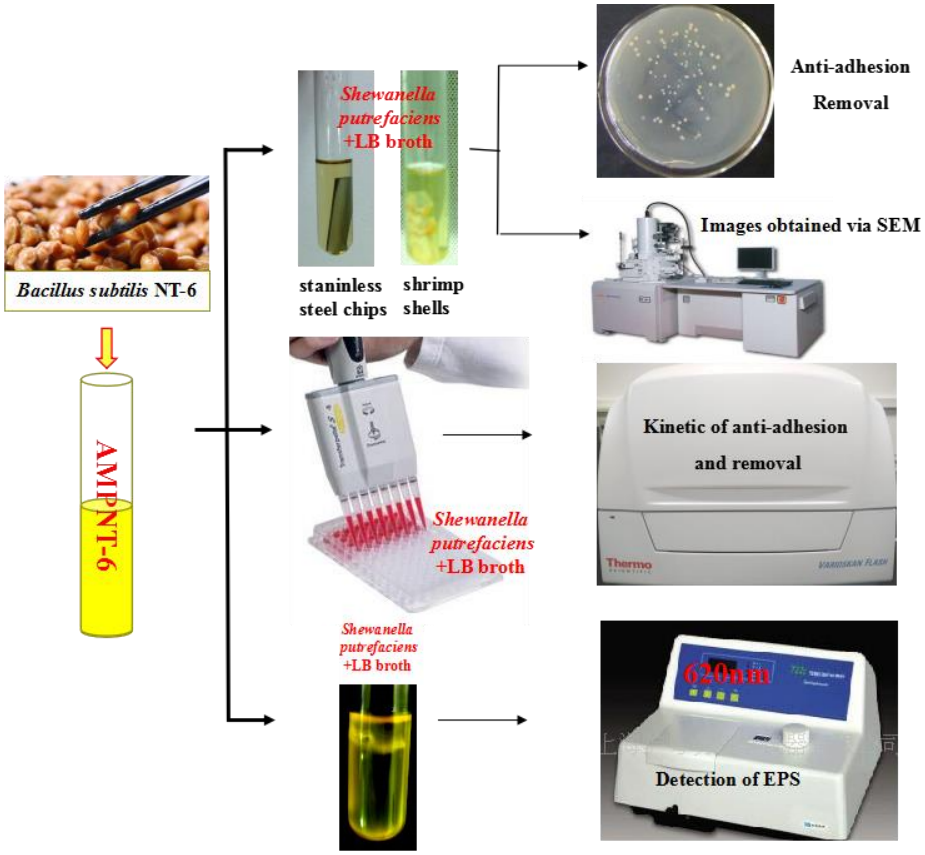


Fig. 6

Graphic abstract



Highlights

- AMPNT-6 was used to control spoilage bacterial biofilm on shrimp shells.
- *S. putrefaciens* biofilm formed more readily on stainless steel than shrimp shells.
- The biofilm of *S. putrefaciens* SL-3 can be removed by AMPNT-6.
- AMPNT-6 displayed a marked dose-dependent anti-adhesive effect > biofilm removal.
- SEM clearly showed AMPNT-6 induced structural damage to biofilms.